INFLAMMATORY GENE RESPONSE IN RAT BRAIN FOLLOWING SOMAN EXPOSURE

Anthony J. Williams[a], Rossana Berti[a], Changping Yao[a], Rebecca A. Price[a], Luisa C. Velarde[a], Irwin Koplovitz[b], Susan M. Shulz[b], Frank C. Tortella[a], and Jitendra R. Dave[a]

[a]Walter Reed Army Institute of Research, Div. Neurosciences, Silver Spring, MD 20910-7500, and [b]U.S. Army Medical Research Institute of Chemical Defense, Basic Assessment Branch, Aberdeen Proving Ground, MD 21010-5425

ABSTRACT

This study reports an upregulation of the neuro-inflammatory gene response using quantitative RT-PCR following soman exposure in rats. Male Sprague-Dawley rats were pre-treated with HI-6 (125 mg/kg, i.p.) and exposed 30 min later to 1.6x LD_{50} of soman (180 µg/kg, s.c.) followed at 1 min by atropine (4 mg/kg, i.m.). Initially, a significant upregulation of TNF-α and VCAM-1 mRNA levels were measured 2 h post-exposure followed at 6 h by IL-1β, IL-6, E-selectin, and ICAM-1 with resolution by 24-48 h. In conclusion, an acute upregulation of the inflammatory gene response is activated following soman exposure and may be involved in soman-induced brain injury.

INTRODUCTION

Despite being prohibited by the Geneva protocol of 1925, nerve agents such as organophosphates remain a primary concern during military engagement. Several countries possess and are capable of delivering these agents against civilian and/or military targets [5]. Nerve agents such as soman, sarin, tabun, GF, and VX can rapidly induce respiratory arrest, incapacitating or killing exposed victims. Other delayed effects of organophosphate poisoning also occur, including damage to the central nervous system (due to brain seizures) leading to death, neurological impairment and/or neuropsychological disorders [12, 15]. Recent military conflicts including the Iran-Iraq war (1980-1988) portrayed the effective use of these chemical nerve agents as a combat weapon.

Following a neurotoxic insult, a local and peripherally recruited neuro-inflammatory response can be induced in the brain despite being normally isolated from the peripheral immune system via the blood brain barrier (BBB). Although the role of brain inflammation has not been definitively defined as a neuropathic process [7], brain inflammation has been proposed to be injurious to the brain due to activation of delayed cell death mechanisms[9, 18]. An acute inflammatory response has been documented in a variety of brain injury or disease states such as multiple sclerosis, Alzheimer's dementia, cerebrovascular disease and brain trauma, which are also associated with a progressive

			Form Approved
Report Documentation Page			*Form Approved* *OMB No. 0704-0188*

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE **01 OCT 2005**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**

4. TITLE AND SUBTITLE **Inflammatory Gene Response In Rat Brain Following Soman Exposure**	5a. CONTRACT NUMBER
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Army Institute of Research, Div. Neurosciences, Silver Spring, MD 20910-7500**	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**UU**	**7**	

neuronal pathology. Importantly, anti-inflammatory treatments of these types of injuries are being explored as therapeutic strategies against the induced cell death [9, 13, 14, 19].

The aim of this study was to evaluate the acute inflammatory reaction to soman exposure to provide a basis for evaluating the potential efficacy of anti-inflammatory agents to help relieve soman induced brain-cell injury (SIBI). In this study we evaluated the inflammatory response of the rat brain to the nerve agent soman as characterized by the upregulation of several inflammatory cytokines and cell adhesion molecules (CAMs). Male Sprague Dawley rats (Charles River Labs, Wilmington, MA) weighing 250-300g were maintained in animal rooms at $21 \pm 2°C$ with $50 \pm 10\%$ humidity. Food and water were provided before and after all procedures and animals were kept on a 12-h light/dark full spectrum lighting cycle with no twilight. Rats were pre-treated with the oxime HI-6 (1-2-hydroxy-iminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino-2-oxapropane dichloride) dissolved in sterile water (125 mg/kg, i.p.). Thirty min later rats were exposed to a 1.6x LD_{50} dose of soman (pinacolyl methyl-phosphonofluoridate, 180 μg/kg, s.c.) followed at 1 min by treatment with atropine methyl nitrate dissolved in sterile water (4 mg/kg, i.m.). Vehicle treated animals were administered an identical treatment protocol except saline (s.c.) was substituted for soman. This same soman exposure protocol has been previously reported to induce SIBI in rats and is used to replicate a severe but survivable injury in a field soldier [8]. Following exposure brain tissue was isolated bilaterally from the hippocampus, thalamus, and piriform cortex at 2, 6, 24, or 48 h for mRNA analysis (4-5 rats per group).

Tissue samples were homogenized and total RNA was extracted from the tissue as previously described [2]. Reverse transcription reactions were carried out using an RNA PCR Core Kit (Perkin Elmer, Branchburg, NJ) on a DNA Thermal Cycler 480 (Perkin Elmer). PCR primers and TaqMan™ probes were designed using Primer Express 1.0 Software program (Perkin-Elmer) and synthesis was performed by Applied Biosystem, CA. The primer and probe sequences for ribosomal protein 32 (rpL32), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), and E-selectin have been previously reported [2]. RT-PCR, as described previously [2], was performed using the TaqMan™ Universal PCR Master Mix, 100 nM of both primers and probe and 1μL of cDNA, on the ABI PRISM 7700 Detection System (Applied Biosystems, Foster City, CA). All data is presented as the mean ± standard error of the mean (n = 4-5 for each group). The mRNA levels were normalized to the corresponding rpL32 level for each sample. Normalization of the data to rpl32 was used to control for slight variation between animals and is commonly used "housekeeping gene" [2]. Statistical analysis of the changes in mRNA level over time for each gene was evaluated by ANOVA followed by Dunnett's post-hoc analysis to compare each post-exposure time point to the sham-treated group.

All animals were visually observed for signs of soman toxicity post-exposure. A total of two animals that did not exhibit signs of exposure (i.e. seizures, salivation, lacrimation of eyes, hypoactivity, ataxia and muscular fasciculations) were excluded from the study. In general, mRNA levels of all inflammatory genes tested were transiently upregulated following exposure to soman with a similar expression profile in each of the three isolated brain regions. At 2 h post-exposure both TNF-α and VCAM-1 were upregulated followed by a delayed upregulation at 6 h by IL-1β, E-selectin, IL-6, and

ICAM-1. In all brain regions mRNA levels returned to within normal (sham–treated) limits by 6-48 h post exposure for every gene tested. Levels of *rpL32* mRNA (control gene) did not significantly change throughout the course of the recovery period.

An acute and transient upregulation of all inflammatory genes was measured in soman-exposed animals. Two hours post-injury mRNA levels of *TNF*-α were dramatically increased by 154 fold in the piriform cortex (Table 1), 58 fold in hippocampal (Table 2), and 29 fold in thalamic tissues (Table 3). Expression levels of *IL-1*β were increased post-exposure but did not reach significantly increased levels until 6 h post-exposure with increases of 55 fold in the piriform cortex (Table 1) and 45 fold in the hippocampus (Table 2). Increases of *IL-1*β in thalamic tissue were not as dramatic (max = 21 fold) and never reached statistically significant levels (Table 3). Expression levels of *IL-6* were increased post-exposure with significantly increased levels at 6 h post-exposure of 119 fold in the piriform cortex (Table 1), 554 fold in hippocampal (Table 2), and 60 fold in thalamic tissues (Table 3). Two hours post-injury significant increases in VCAM-1 were evident in all brain regions studied (2.6 fold increase in the piriform cortex (Table 1), 2.9 fold in the hippocampus (Table 2), and 2.2 fold in the thalamus (Table 3)). Expression levels of *ICAM-1* were increased post-exposure but did not reach significance until 6 h post-exposure with increases of 17 fold in the piriform cortex (Table 1), 13 fold in hippocampal (Table 2), and 19 fold in thalamic tissues (Table 3). Expression levels of *E-Selectin* were marginal at all times post injury except at 6 h where dramatic and significant increases of 70 fold in the piriform cortex (Table 1), 11 fold in hippocampal (Table 2), and 39 fold in thalamic tissues (Table 3) were measured.

Table 1. QRT-PCR analysis of changes in inflammatory gene expression out to 48 h following acute soman exposure in tissue isolated from the ***piriform cortex*** of rats. Relative mRNA levels are given as the mean ± SEM (n = 4-5/group). Asterisks (*) indicate P < 0.05 as compared to sham group (ANOVA followed by Dunnett's post-hoc analysis).

gene	sham	2h	6h	24h	48h
E-selectin	1.17±0.73	0.90±0.24	82.1±29.9*	3.24±2.27	1.19±0.54
IL-6	1.22±0.57	80.5±44.7	306±130*	109±89	1.29±0.44
IL-1β	2.57±0.38	22.0±8.0	140±70*	8.23±3.66	2.40±0.79
TNF-α	2.32±0.41	359±118*	17.9±4.8	2.73±0.58	1.68±0.52
ICAM-1	21.3±2.5	118±42	371±90*	44.7±15.7	26.2±4.5
VCAM	883±237	2270±265*	730±137	746±307	141±46

Table 2. QRT-PCR analysis of changes in inflammatory gene expression out to 48 h following acute soman exposure in tissue isolated from the ***hippocampus*** of rats. Relative mRNA levels are given as the mean ± SEM (n = 4-5/group). Asterisks (*) indicate P < 0.05 as compared to sham group (ANOVA followed by Dunnett's post-hoc analysis).

gene	sham	2h	6h	24h	48h
IL-1β	2.1±0.92	13.3±5.4	43.4±9.9	52.8±31.4	16.1±5.7
IL-6	3.53±1.90	83.4±50.8	210±41*	78.7±43.4	12.6±5.1
ICAM-1	9.61±2.65	77.3±36.2	180±33*	38.9±18	46.1±7.0
E-selectin	0.490±0.130	1.41±0.55	19.0±3.9*	3.80±1.90	8.92±3.88
VCAM	968±196	2172±403*	356±425	675±199	449±100
TNF-α	3.84±1.40	111±51*	31.6±24.2	8.2±3.2	10.8±3.6

Table 3. QRT-PCR analysis of changes in inflammatory gene expression out to 48 h following acute soman exposure in tissue isolated from the ***thalamus*** of rats. Relative mRNA levels are given as the mean ± SEM (n = 4-5/group). Asterisks (*) indicate P < 0.05 as compared to sham group (ANOVA followed by Dunnett's post-hoc analysis).

gene	sham	2h	6h	24h	48h
E-selectin	2.40±1.67	1.55±0.49	27.4±7.5	2.26±1.17	1.59±0.96
IL-1β	0.960±0.260	9.91±2.48	43.5±4.4*	6.86±3.64	1.90±0.84
IL-6	0.240±0.090	66.1±27.5	133±33*	97.6±73.9	0.830±0.420
TNF-α	2.19±0.66	127±72	30.3±2.1	2.38±0.62	1.93±0.42
ICAM-1	11.3±2.0	68.9±25.1	151±23*	29.4±11.9	29.2±6.1
VCAM	930±252	2652±399*	486±80	741±306	163±51

The histopathological response to exposure with the irreversible cholinesterase inhibitor soman has been well documented [6, 11, 20]. Importantly, even with currently available treatments for soman exposure acute brain lesions can occur post-exposure. Vulnerable brain regions include the thalamus, hippocampus, and piriform cortex. Brain pathology includes progression of edema and inflammatory cell activation followed at later time points by the presence of necrotic cell death. The soman induced inflammatory response includes the upregulation of glial fibrillary acidic protein mRNA levels and the associated activation of astrocytes and microglia, predominately in hippocampus and piriform cortex [1, 20]. By 4-8 h post-exposure the presence of macrophages in the brain have also been reported [20]. Active inflammation is promoted by the production of molecules such as cytokines, capable of being produced by a wide variety of cells [17]. Production of cytokines can be initiated following a variety of neurotoxic insults [17]. This upregulation is also true for soman exposure as indicated in this study. Between 2-6 h post-soman exposure we measured a 20 to over 550 fold increase in the cytokines *TNF-α*, *IL-1*β, and *IL-6* in the three brain regions studied. Cytokines have a wide range of biological activity and play not only a neurotoxic role during the early stages of injury but may also be involved in repair processes during the recovery period [17]. Cytokines such as IL-6, TNF-α and IL-1β can be particularly toxic, however, by initiating

activation of apoptotic-associated caspases and production of reactive nitrogen species, which have been linked to the induction of cell death [17, 18].

Cytokines have also been associated with the recruitment of circulating inflammatory cells by upregulating the expression of CAMs [9]. In this study we verified the early but mild (2-3 fold) upregulation of *VCAM-1* at 2 h post-exposure as compared to the higher increases in *ICAM-1* (13-19 fold) and *E-selectin* (11-70 fold) at 6 h post-exposure. Both *ICAM-1* and *VCAM-1* have been shown to be upregulated in experimental encephalomyelitis [4]. Similarly, *ICAM-1* and *E-selectin* are transiently upregulated following focal ischemic brain injury in rats [2]. The upregulation of CAMs following injury or toxic insult to the brain has been associated with the recruitment of peripheral inflammatory cells including neutrophils and macrophages into the brain that may aggravate the inflammatory response [4, 16]. Importantly, interruption of the CAM-induced recruitment of peripheral inflammatory cells has been shown to correlate with increased neuroprotective outcome following experimental brain injury [2, 16] and allergic encephalomyelitis [4]. Based on the current study, the increase in inflammatory gene mRNA levels was 2-3 fold higher in the piriform cortex as compared to the other brain regions studied. The increased gene expression levels in the piriform cortex following soman exposure may indicate an enhanced inflammatory cell infiltration into this brain region, although this remains to be verified.

Brain injury due to soman exposure has been associated with and, in fact, dependent on the occurrence of brain seizure activity [10, 12]. In the case of seizure development, administration of anticonvulsants such as diazepam is therapeutic for controlling brain seizures and reduces associated neuropathology [12]. Although treatment with anticonvulsants such as diazepam is highly effective in reducing SIBI, the therapeutic treatment window only extends to about 40 min post-exposure [12]. The inflammatory response is generally delayed for several hours following many types of neuropathies including those due to infection, autoimmune, and ischemic or traumatic injury [17], which may allow for an extended therapeutic treatment window. As indicated from our data, the peak inflammatory response does not occur for 2-6 hours post-injury. We have recently reported a similar inflammatory gene expression profile following transient ischemic brain injury in rats [2]. Furthermore, we reported a 10 h delayed treatment window with the anti-inflammatory proteasome inhibitor MLN519 in this same ischemic brain injury model [3]. Thus, treatment of SIBI with anti-inflammatory agents may provide and extended therapeutic treatment window as compared to currently available treatment protocols.

CONCLUSIONS

In conclusion, these results indicate that soman induces a rapid and strong upregulation of the inflammatory gene response of the brain, which may be a potential target for post-exposure treatment with anti-inflammatory compounds. In particular, soman exposure induces an early activation of several cytokines and CAMs that peak in expression by 2-6 h and returned to within pre-exposure levels by 24-48 h. Future studies are being planned to evaluate inflammatory cell response and protein levels of these respective genes utilizing histopathological and immunohistochemical techniques.

REFERENCES

[1]   Baille-Le Crom, V., Collombet, J.M., Carpentier, P., Brochier, G., Burckhart, M.F., Foquin, A., Pernot-Marino, I., Rondouin, G. and Lallement, G., Early regional changes of GFAP mRNA in rat hippocampus and dentate gyrus during soman-induced seizures, Neuroreport, 7 (1995) 365-369.

[2]   Berti, R., Williams, A., Moffett, J., Hale, S., Velarde, L., Elliott, P., Yao, C., Dave, J. and Tortella, F., Real time PCR mRNA analysis of the inflammatory cascade associated with ischemia reperfusion brain injury, J. Cereb. Blood Flow Metab., 22 (2002) 1068-1079.

[3]   Berti, R., Williams, A., Moffett, J., Velarde, L., Cunningham, B., Elliott, P., Adams, J., Yao, C., Dave, J. and Tortella, F., Brain Trauma and the inflammatory process: use of a novel neuroprotective drug targetting NF-kB, Soc. Neurosci. Abstr., 28 (2002).

[4]   Brown, K., Factors modifying the migration of lymphocytes across the blood-brain barrier., Int. Immunopharmacol., 1 (2001) 2043-2062.

[5]   Brown, M.A. and Brix, K.A., Review of health consequences from high-, intermediate- and low-level exposure to organophosphorus nerve agents, J. Appl. Toxicol., 18 (1998) 393-408.

[6]   Carpentier, P., Lambrinidis, M. and Blanchet, G., Early dendritic changes in hippocampal pyramidal neurones (field CA1) of rats subjected to acute soman intoxication: a light microscopic study, Brain Res., 541 (1991) 293-299.

[7]   Emerich, D., Dean, R. and Bartus, R., The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct?, Exp. Neurol., 173 (2002) 168-181.

[8]   Filbert, M.G., Forster, J.S., Smith, C.D. and Ballough, G.P., Neuroprotective effects of HU-211 on brain damage resulting from soman- induced seizures, Ann N. Y. Acad. Sci., 890 (1999) 505-514.

[9]   Frijns, C.J. and Kappelle, L.J., Inflammatory cell adhesion molecules in ischemic cerebrovascular disease, Stroke, 33 (2002) 2115-2122.

[10]   Lallement, G., Pernot-Marino, I., Baubichon, D., Burckhart, M.F., Carpentier, P. and Blanchet, G., Modulation of soman-induced neuropathology with an anticonvulsant regimen, Neuroreport, 5 (1994) 2265-2268.

[11]   Lemercier, G., Carpentier, P., Sentenac-Roumanou, H. and Morelis, P., Histological and histochemical changes in the central nervous system of the rat poisoned by an irreversible anticholinesterase organophosphorus compound, Acta Neuropathol., 61 (1983) 123-129.

[12]   McDonough, J.H., Jr., Dochterman, L.W., Smith, C.D. and Shih, T.M., Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of drug treatment, Neurotox., 16 (1995) 123-132.

[13]   Phillips, J., Williams, A., Adams, J., Elliott, P. and Tortella, F., Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia., Stroke, 31 (2000) 1686-1693.

6

[14]   Piehl, F. and Lidman, O., Neuroinflammation in the rat--CNS cells and their role in the regulation of immune reactions., Immunol. Rev., 184 (2001) 212-225.

[15]   Schenker, M., Louie, S., Mehler, L., Albertson, T., Pesticides. In W. Rom (Ed.), Environmental Occupational Medicine, 3rd ed., Lippincott-Raven Publishers, Philadelphia, 1998, pp. 1157-1163.

[16]   Takeda, H., Spatz, M., Ruetzler, C., McCarron, R., Becker, K. and Hallenbeck, J., Induction of mucosal tolerance to E-selectin prevents ischemic and hemorrhagic stroke in spontaneously hypertensive genetically stroke-prone rats., Stroke, 33 (2002) 2156-2164.

[17]   Wang, C. and Shuaib, A., Involvement of inflammatory cytokines in central nervous system injury., Prog. Neurobiol., 67 (2002) 161-172.

[18]   Wang, J., Asensio, V. and Campbell, I., Cytokines and chemokines as mediators of protection and injury in the central nervous system assessed in transgenic mice., Curr. Top. Microbiol. Immunol., 265 (2002) 23-48.

[19]   Williams, A.J., Hale, S.L., Moffett, J.R., Dave, J.R., Elliott, P.J., Adams, J. and Tortella, F.C., Delayed Treatment With MLN519 Reduces Infarction and Associated Neurologic Deficit Caused by Focal Ischemic Brain Injury in Rats via Antiinflammatory Mechanisms Involving Nuclear Factor-kappaB Activation, Gliosis, and Leukocyte Infiltration, J. Cereb. Blood Flow Metab., 23 (2003) 75-87.

[20]   Zimmer, L.A., Ennis, M. and Shipley, M.T., Soman-induced seizures rapidly activate astrocytes and microglia in discrete brain regions, J. Comp. Neurol., 378 (1997) 482-492.

FOOTNOTE

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85-23. Earlier versions of this data have been previously reported (Williams et al., Neurosci. Lett., 349 (2003) 147-150). The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3), AR 360-5.